

Picomolar inhibition of cholera toxin by a pentavalent ganglioside GM1os-calix[5]arene†

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Cholera toxin (CT), the causative agent of cholera, displays a pentavalent binding domain that targets the oligosaccharide of ganglioside GM1 (GM1os) on the periphery of human abdominal epithelial cells. Here, we report the first GM1os-based CT inhibitor that matches the valency of the CT binding domain (CTB). This pentavalent inhibitor contains five GM1os moieties linked to a calix[5]arene scaffold. When evaluated by an inhibition assay, it achieved a picomolar inhibition potency ($IC_{50} = 450$ pM) for CTB. This represents a significant multivalency effect, with a relative inhibitory potency of 100 000 compared to a monovalent GM1os derivative, making GM1os-calix[5]arene one of the most potent known CTB inhibitors.

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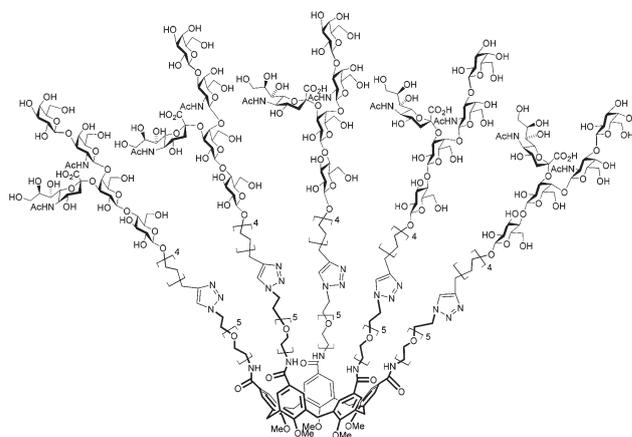
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Introduction

Cholera still represents a serious health problem in areas of the developing world where there is a lack of clean water and proper sanitation. In 2012, the World Health Organization estimated that annually 3–5 million cholera cases occur that result in more than 100 000 deaths.¹ Although several treatments exist for cholera,² resistance development and mutations in the causative pathogen mean that efforts made to better understand the disease pathogenesis and develop new treatments are crucial.^{3,4} The symptoms of cholera are caused by cholera toxin (CT), which is produced by the *Vibrio cholerae* bacterium. CT is a member of the AB₅ toxin family that contains a pentameric binding domain (CTB) for recognition and binding to cell surfaces.⁵ The natural target ligand for CTB is the glycosphingolipid ganglioside GM1, on cellular membranes of the infected hosts' intestinal epithelial surface. CTB can bind five GM1 saccharide epitopes simultaneously with the terminal Gal- and the Neu5Ac carbohydrate units of the ganglioside as the major contributors to the binding.^{6,7} The adhesion of CTB to ganglioside GM1 on cell surfaces is the

prerequisite for endocytosis of the toxic enzymatically active A subunit of CT, and the ensuing severe clinical symptoms.⁸ One avenue in cholera research is to study the binding of CTB to GM1 and to develop CTB inhibitors that might prevent CT from binding the hosts' cell surface and thereby also the development of cholera. Here, we present the second of two examples of a pentavalent GM1os-based inhibitor for CTB, GM1os-calix[5]arene (**1**; Fig. 1). In the previous paper in this issue, we also reported on pentavalent inhibitors of CTB based on a GM1os-presenting corannulene scaffold. In the past, several studies have focused on the development of multivalent glycosylated inhibitors for CTB based on ganglioside GM1.⁹ It is noteworthy that in none of these studies, inhibitors were investigated with a pentavalent structure that matches the pentavalent structure of CTB. On the other hand, pentavalent

Fig. 1 Developed CTB inhibitor: penta-GM1os-calix[5]arene (**1**).^aLaboratory of Organic Chemistry, Wageningen University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands. E-mail: Han.Zuilhof@wur.nl^bUniversità degli Studi di Parma, Dipartimento di Chimica, Parco Area delle Scienze 17/a, 43124 Parma, Italy. E-mail: Casnati@unipr.it^cInstitute for Biological Sciences, National Research Council Canada, 100 Sussex Drive, Ottawa, Ontario, Canada^dDepartment of Chemical and Materials Engineering, King Abdulaziz University, Jeddah, Saudi Arabia

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cyclens^{10,11} and cyclic peptides¹² have been described as CTB inhibitors, but those contained only the much simpler galactose epitope likely to get around the difficulty to obtain sufficient tailor-made GM1os. Therefore, also GM1 mimics have been used, *e.g.* Thompson and Schengrund described poly(propylene) imine dendrimers that present the Gal β 1-3GalNAc β 1-4(Neu5Ac α 2-3)Gal β -epitope of GM1,¹³ with IC₅₀ values for the tetravalent and octavalent dendrimers of 7 and 3 nM, respectively. Bernardi *et al.* published a series of GM1-mimics (pseudo-GM1), in which the residues in the GM1os that are not essential for binding were replaced by a conformationally restricted cyclohexane-diol and the Neu5Ac-unit was substituted by various α -hydroxy acids.^{14,15} When attached to multivalent dendritic structures,¹⁶ the relative inhibitory potency (RIP) values per mimic unit of the tetravalent and octavalent inhibitors were 111 and 55, respectively. Interestingly, when the same mimic was linked to a divalent calix[4]-arene scaffold,¹⁷ a 4000-fold enhancement in binding efficiency was achieved compared to the monovalent pseudo-GM1. These data suggested to us that the calixarene macrocycle, from which the binding inhibitors are projected, could be a promising multivalent scaffold¹⁸⁻²⁰ to design CTB inhibitors with improved efficiency. In collaboration with the group of Pieters, we previously published divalent, tetravalent, and octavalent dendritic structures decorated with GM1os.^{21,22} For the octavalent compound the unprecedentedly low IC₅₀ value of 50 pM was observed with an RIP of 17 500 per arm compared to its monovalent counterpart. However, its mismatched valency compared to CTB prompted us to investigate a pentavalent scaffold as core structure that when decorated with GM1os has the potential to form 1:1 inhibitor-CTB complexes. The current paper presents the convergent synthesis of the first, water-soluble, pentavalent CTB inhibitor (**1**), which

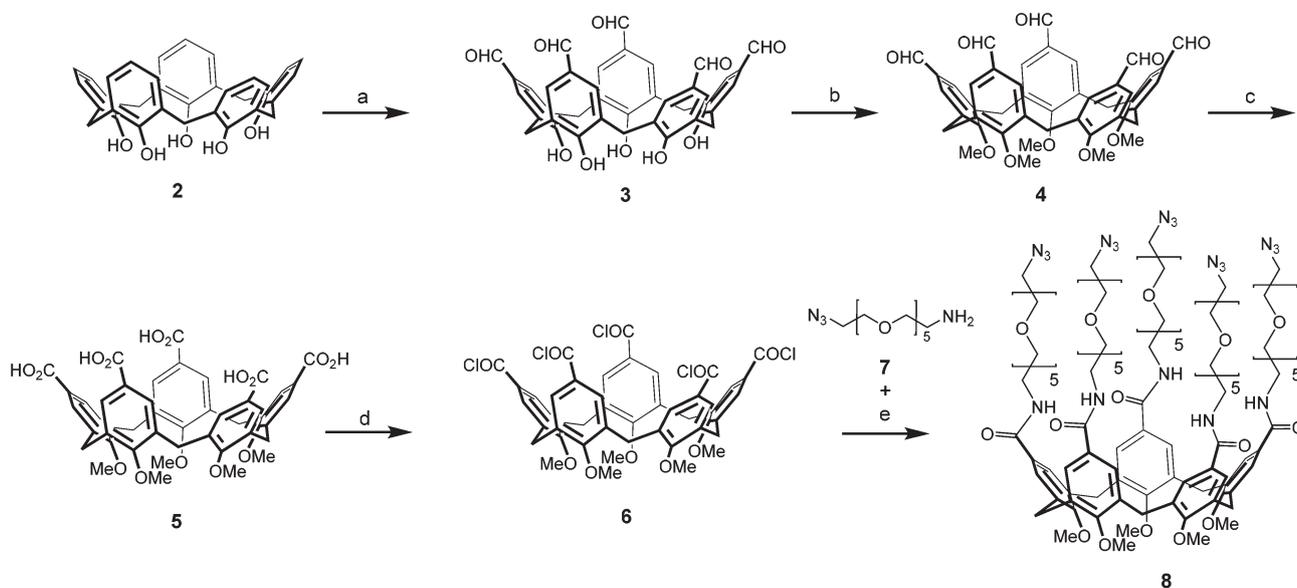
was made by coupling five GM1os units to a calix[5]arene scaffold.

Results and discussion

We designed a 5-fold symmetric calix[5]arene as a pentavalent scaffold structure. This calix[5]arene (Fig. 1) presents small methoxy groups at the lower rim, which confer a high conformational flexibility to the macrocyclic structure.²³ The upper rim of the calixarene inhibitor is decorated with the GM1 penta-saccharide separated from the macrocyclic core by appropriate linkers. Fan and coworkers¹² have demonstrated that an optimal linker length is vital for the potency of a synthetic multivalent inhibitor. For the calix[5]arene, described here, a 31 atom-containing linker was chosen. This should allow the simultaneous interaction of the five GM1os units with the five B-subunits of a single toxin.⁵

The route towards our target (**1**) started with the synthesis of the pentavalent scaffold that began with the preparation of the known *p*-*tert*-butyl-calix[5]arene.²⁴ This product was converted into *p*-H-calix[5]arene **2**²⁵ by following literature procedures. Next, penta-aldehyde **3** was obtained in 57% yield from **2** by exploiting the Duff formylation reaction.^{26,27} Compound **3** was subsequently methylated at the lower rim by using CH₃I and K₂CO₃ in acetonitrile affording the penta-methoxy-calix[5]arene **4** in 68% yield (Scheme 1).

Oxidation of **4** with NaClO₂ and NH₂SO₃H produced the penta-carboxylic acid **5**. The unsymmetrically substituted azido-penta-(ethyleneglycol)-amine **7** was synthesized from hexa-ethylene glycol by ditosylation, substitution to the diazide, and finally selective Staudinger reduction of one azide.^{28,29} Initial attempts to condense amine **7** with the carboxylic acids



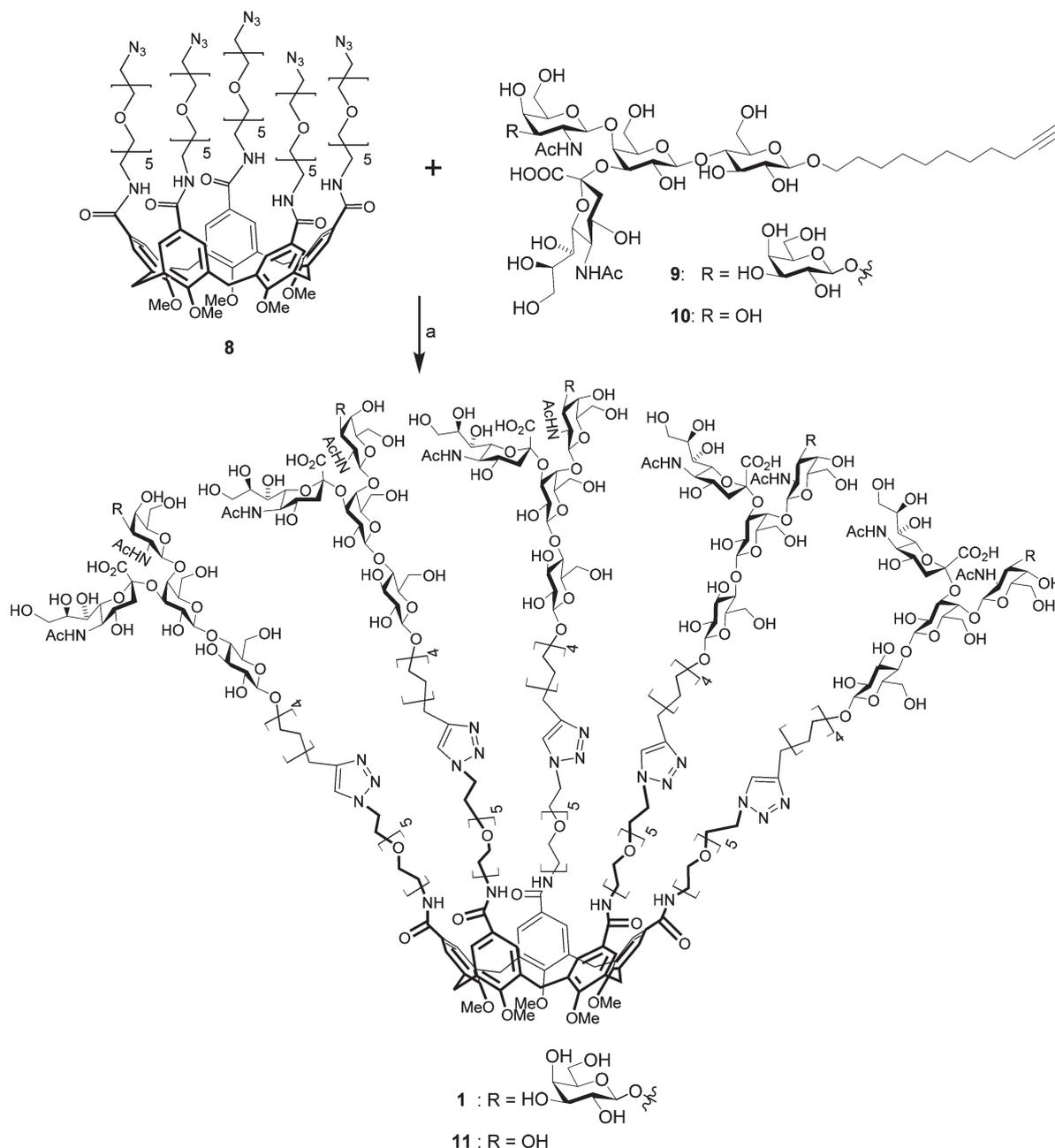
Scheme 1 Synthesis of the penta-azido-calix[5]arene (**8**) scaffold. Reagents and conditions: (a) HMTA, CF₃COOH, reflux, N₂, 5 days, 57%; (b) CH₃I, K₂CO₃ CH₃CN, reflux, N₂, 20 h, 68%; (c) NaClO₂, NH₂SO₃H, (CH₃)₂CO, CHCl₃, H₂O, rt, 24 h, 79%; (d) (COCl)₂, dry CH₂Cl₂, N₂, rt, 18 h, quant.; (e) Et₃N, dry CH₂Cl₂, N₂, rt, 20 h, 44%.



in **5** using HBTU resulted in difficult purification and low yields (~20%) of **8**. However, when this spacer (**7**) was attached to calix[5]arene **5** via penta-acyl chloride intermediate **6** it provided penta-azido-calix[5]arene **8** in a 44% yield.

With the calix[5]arene (**8**) scaffold in hand we proceeded to the next stage, attaching five GM1 oligosaccharides. We chose the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction to achieve this, which meant a GM1os derivative with a terminal alkyne was required. This C₁₁-alkyne-terminated GM1os **9** (Scheme 2) was made *via* a chemo-enzymatic procedure previously reported by us,³⁰ which allowed the

production of **9** on gram scale. Compound **9** was subsequently “clicked” to scaffold **8** under standard CuAAC conditions in H₂O while exposed to microwave irradiation to successfully provide our crude target inhibitor **1**. With our target penta-valent GM1os-calix[5]arene **1** in hand, in order to properly assess the role of the GM1os in inhibitor **1**, we also set out to synthesize derivatives of **1** containing fragments of the GM1os to use for comparison in the biological assays. The first of these was penta-GM2os-calix[5]arene (**11**) that lacks the terminal galactose epitope compared to the GM1os. We synthesized **11** using the same CuAAC reaction conditions from scaffold **8** and



Scheme 2 Synthesis of GM1os-calix[5]arene (**1**) and GM2os-calix[5]arene (**11**); (a) CuSO₄·5H₂O, sodium ascorbate, Triton X-100, CH₃OH, H₂O, MW (150 W), 80 °C, 1 h; 51% **1**, 59% **11**.



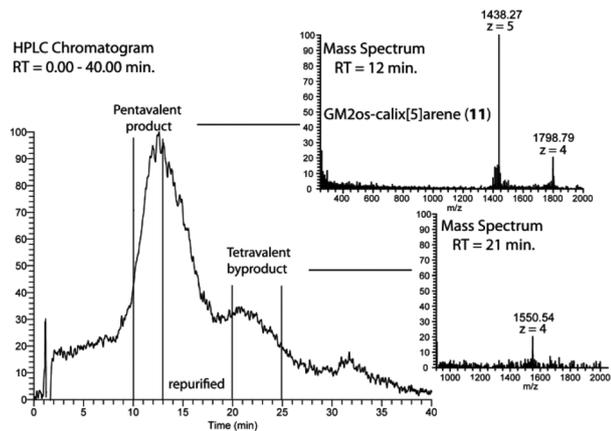


Fig. 2 LCMS trace of the purification of GM2-calix[5]arene **11**. (left) Chromatogram of the purification on a reversed phase column (see experimental section for details). (right) Mass spectra for two fractions, the pentavalent product **11** at RT = 12 min, and the tetraivalent byproduct at 21 min.

a chemo-enzymatically produced alkyne-terminated C_{11} -linked GM2os sugar (**10**).

With both our target **1** and its derivative **11** in hand as crude products, we investigated what purification method would be suitable for these large complex molecules. An initial purification by size exclusion chromatography (SEC) efficiently removed an excess of alkyne-terminated GM1os **9** and GM2os **10**, respectively. However, the crude products both also contained a minor amount of tetraivalent byproducts as could be clearly seen with mass spectrometry and their separation proved to be quite challenging. Initial attempts to separate these by aqueous HPLC GPC failed, but after extensive optimization, reversed phase HPLC purification proved the most successful for this final purification step (see experimental section for details). Fig. 2 shows a typical HPLC chromatogram for the separation of both the GM1os- and GM2os-calix[5]-arenes. Despite attempts to improve the moderate resolution, the purification remained quite complicated because of long elution times (up to 40 minutes per run) and high affinity of the products with the column material. Collection of small fractions in a specific retention time window over multiple HPLC injections and subsequent lyophilization resulted indeed in pure pentavalent GM1os- and GM2os-calix[5]arenes (**1** and **11**) as shown by HR-MS and NMR analyses. The other impure fractions that also contained **1** or **11** were collected, pooled, lyophilized and re-injected to achieve optimal yields. Attempts were made to isolate the tetraivalent byproducts, but insufficient amounts could be obtained for further analyses.

Besides the GM2os containing calixarene (**11**), we also prepared two further derivatives of **1** that contained fragments of GM1os, a pentavalent β -galactoside- (**16**) and β -lactoside-calix[5]arene (**17**) (Scheme 3). These more simple carbohydrates enabled a modified synthesis procedure that circumvented the potentially challenging HPLC purification, as encountered for compounds **1** and **11**. The coupling was also performed by employing the microwave-assisted CuAAC reaction on penta-

azido-calixarene scaffold **8**, but instead of using the deprotected carbohydrates, acetyl-protected galactoside **12**, and lactoside **13** were reacted. The resulting products could now be purified by normal phase silica gel chromatography. The acetyl-protected **14** and **15** were deprotected by employing standard Zemplén³¹ conditions to obtain galactoside-calix[5]arene **16**, and lactoside-calix[5]arene **17**, respectively, which did not require further purification after work-up.

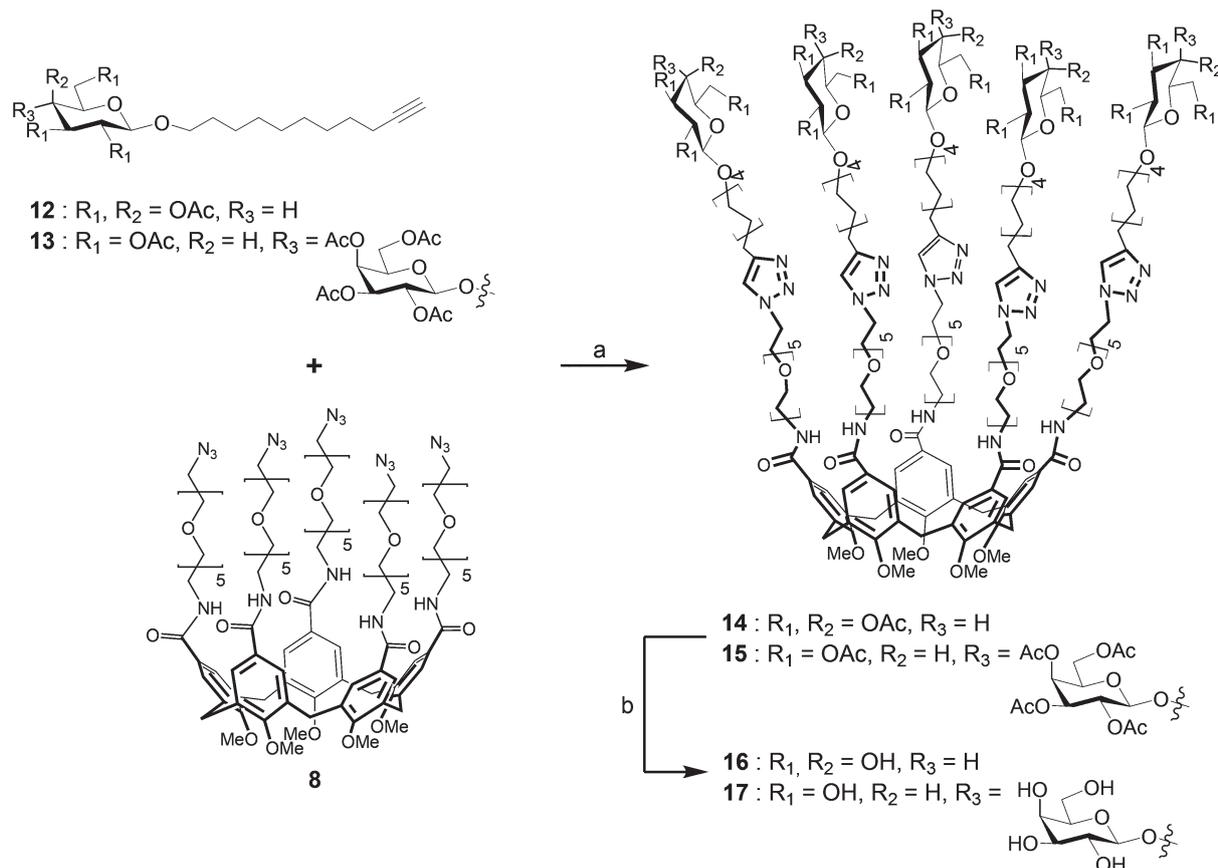
Finally, in order to properly determine the multivalency effect of the interaction of **1** with CTB in our biological assays, we also synthesized the monovalent GM1os derivative **20** (Scheme 4). This was achieved by first *in situ* generation of the acyl chloride of commercially available 4-methoxybenzoic acid with oxalyl chloride and, subsequently, reacting this with amino-azide **7**, yielding azide **19** in 20% over two steps. Again, by employing the microwave-assisted CuAAC reaction on alkyne-terminated **9** and azide **19**, GM1os-monomer **20** was obtained in a reasonable yield (49%).

The inhibitory potency of the four pentavalent compounds (**1**, **11**, **16**, and **17**) was determined by ELISA experiments. In the assays, the ability of **1**, **11**, **16** and **17** to inhibit the binding of HRP-labeled CTB was measured in competition with the natural ligand ganglioside GM1, which was adsorbed to the well surface of the ELISA plate. GM1os-calix[5]arene **1** showed a high inhibition potency, *i.e.*, a very low IC_{50} value of 450 pM (Fig. 3, Table 1). Comparing the IC_{50} value (44 μ M) of the monovalent control compound (**20**) to that of **1** revealed a 100-thousand increase in inhibitory potency, and an RIP of 20-thousand per arm. Pentavalent inhibitors based on a more rigid corannulene scaffold, which we also report in this issue, inhibited CTB in the nanomolar range.³² Assay results for the GM2os-calix[5]arene **11** confirmed the importance of using GM1os. Lacking only the terminal galactose compared to **1**, it produced an IC_{50} of 9 μ M, which is 20-thousand fold worse compared to **1**. The galactose-terminated (**16**) and lactose-terminated (**17**) calix[5]arenes displayed a higher inhibition concentration than their solubility in the assay medium, and their IC_{50} could therefore only be determined as being >1 mM (Table 1).

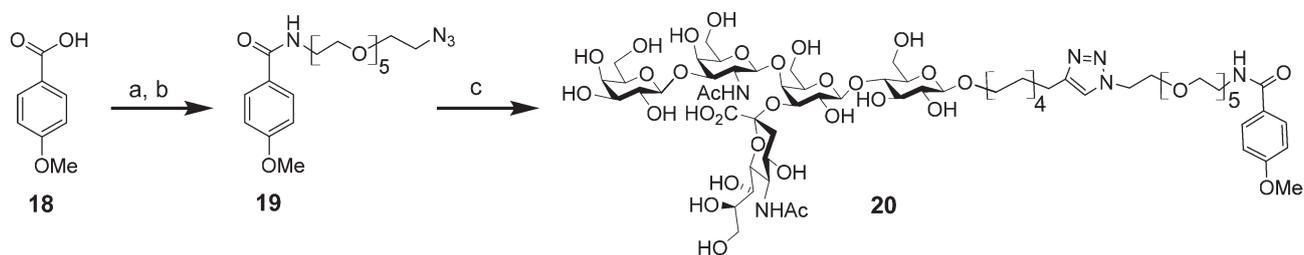
Conclusions

In summary, we here report the synthesis and initial biological evaluation of the first known example of a pentavalent GM1os-based inhibitor of cholera toxin that matches the valency of the cholera toxin B-subunit. With an IC_{50} of 450 pM, the pentavalent GM1os-calix[5]arene (**1**) also displays the highest relative inhibitory potency, 20-thousand per arm (compared to **20**), documented thus far for CT inhibitors. We are currently using the here reported convergent synthetic route to further explore the structure-activity-relationship of **1** and improve its potency. Among other issues we are interested in investigating the effect of the length, rigidity and hydrophobicity of the used spacer and restricting the flexibility of the calix[5]arene scaffold to a fixed cone structure.





Scheme 3 Synthesis of galactoside-calix[5]arene (**16**), and lactoside-calix[5]arene (**17**); (a) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sodium ascorbate, DMF, H_2O , MW (150 W), 80°C , 1 h; 67% **14**, 57% **15**; (b) NaOMe – MeOH , 4 h – 18 h, H^+ -resin; 90% **16**, 72% **17**.



Scheme 4 Synthesis of GM1-monomer **20**; (a) $(\text{COCl})_2$, dry CH_2Cl_2 , N_2 , rt, 18 h; (b) Et_3N , dry CH_2Cl_2 , N_2 , rt, 20 h; 20% in two steps; (c) **9**, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, sodium ascorbate, Triton X-100, CH_3OH , H_2O , MW (150 W), 80°C , 1 h; 49% **20**.

Experimental section

General experimental information

All moisture sensitive reactions were carried out under a nitrogen atmosphere, using previously oven-dried glassware. All dry solvents were prepared according to standard procedures, distilled before use and stored over 3 Å or 4 Å molecular sieves. Reagents were obtained from commercial sources and used without further purification unless stated otherwise. Analytical TLC was performed using prepared plates of silica gel (Merck 60 F-254 on aluminium) and then, according to the functional groups present on the molecules, revealed with UV light or

using staining reagents: FeCl_3 (1% in H_2O – CH_3OH 1:1), H_2SO_4 (5% in EtOH), ninhydrin (5% in EtOH), basic solution of KMnO_4 (0.75% in H_2O). Merck silica gel 60 (70–230 mesh) was used for flash chromatography and for preparative TLC plates. ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AV300, Bruker AV400, Bruker DPX400, and Bruker AV600 equipped with cryoprobe spectrometers (observation of ^1H nucleus at 300 MHz, 400 MHz, 600 MHz, respectively, and of ^{13}C nucleus at 75 MHz, 100 MHz, and 151 MHz, respectively). Chemical shifts are reported in parts per million (ppm), calibrated on the residual peak of the solvent, whose values are referred to tetramethylsilane (TMS, $\delta_{\text{TMS}} = 0$), as the internal



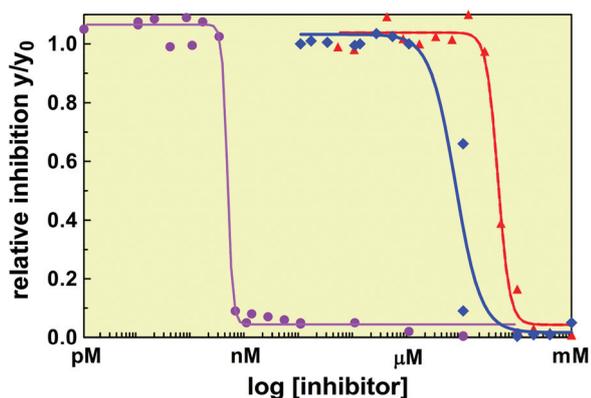


Fig. 3 ● GM1os-calix[5]arene ◆ GM2os-calix[5]arene ▲ GM1os-monomer; Fitted curves of the experimental ELISA inhibition data. For details of the inhibition assays see experimental section.

Table 1 CTB inhibition potency for reported compounds

Entry	Saccharide	Valency	#	IC ₅₀
1	GM1os	5	1	450 pM
2	GM1os	1	20	44 μM
3	GM2os	5	11	9 μM
4	Galactose	5	16	>1 mM
5	Lactose	5	17	>1 mM

standard. ¹³C NMR spectra were performed with proton decoupling. Electrospray ionization (ESI) mass analyses were performed with a Waters spectrometer, while high resolution ESI mass analyses were recorded on a Thermo Scientific Q Exactive spectrometer. Melting points were determined on an Electro-thermal apparatus in closed capillaries. Microwave reactions were performed on a CEM Discovery System reactor running on Discover Application Chemdriver Software v3.6.0. HPLC was performed on an HP 1100 series with a DAP 190–600 nM detector, equipped with a Waters Xterra 100 × 4.6 mm C18 column eluted with isocratic iPrOH–H₂O 35 : 65, and a flow of 0.4 mL min⁻¹, unless stated otherwise. Materials for the ELISA experiments *i.e.* bovine serum albumin (BSA), bovine brain GM1, *ortho*-phenylenediamine (dihydrochloride salt) (OPD), cholera toxin horseradish peroxide (CTB-HRP) conjugate, Tween-20, 30% H₂O₂ solution, sodium citrate, and citric acid were purchased at Sigma Aldrich and used without further modification, phosphate-buffered saline (PBS) 10× concentrate was diluted ten times with demineralized water prior to use. Nunc F96 Maxisorp™ 96-well microtiter plates were used as purchased at Thermo Scientific. The microtiter plates were washed using an automated Denville® 2 Microplate Washer. Optical density (OD) was measured between 1.5 and 0.5 units on a Thermo Labsystems Multiskan Spectrum Reader running on Skanit software version 2.4.2. Data analysis and curve fitting of inhibition experiments were performed on Prism Graphpad software v5.04. Simplified nomenclature proposed by Gutsche³³ is used to name the calix[5]arene compounds.

Compounds 31,32,33,34,35-pentahydroxycalix[5]arene 2,²⁵ 17-azide-3,6,9,12,15-pentaoxaheptadecane-1-amine 7,²⁹ undec-10-ynyl-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside 12,³⁰ undec-10-ynyl-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside 13³⁰ were prepared according to literature procedures.

GM1-calix[5]arene (1)

Calix[5]arene 8 (15.7 mg, 6.93 μmol) was dissolved in 0.5 mL of CH₃OH in a microwave tube. Then the GM1os derivative 9 (59.8 mg, 52.1 μmol), previously synthesized by chemo-enzymatic procedures,^{30,34} was combined with CuSO₄·5H₂O (0.52 mg, 2.1 μmol), sodium ascorbate (0.82 mg, 4.2 μmol), 4 mL of H₂O and a drop of Triton X-100. The mixture was heated at 80 °C by microwave irradiation (150 W) for 60 min. The reaction progression was monitored *via* TLC (eluent: AcOEt–CH₃OH–H₂O–AcOH 4 : 2 : 1 : 0.1) and ESI-MS analyses. The crude mixture was purified *via* size exclusion column chromatography (Sephadex G-15, eluent: H₂O 100%) and HPLC purification (see General information) giving product 1 as a white solid. Yield: 51%. ¹H NMR (600 MHz, D₂O): δ (ppm) 7.61 (s, 5H, H5 triazole); 7.50 (s, 10H, Ar); 4.69 (bs, 5H, H1-GalNAc); 4.43 (d, 5H, *J* = 8.0 Hz, H1-Gal'); 4.44–4.41 (m, 5H, H1-Gal); 4.38–4.37 (m, 5H); 4.32–4.31 (5H, d, *J* = 7.9 Hz, H1-Glc); 4.05–4.02 (m, 15H); 3.94–3.92 (m, 5H, H2-GalNAc); 3.86–3.60 (m, 105H); 3.60–3.45 (m, 80H); 3.46–3.33 (m, 75H); 3.26–3.24 (m, 5H, H2-Gal); 3.18–3.15 (m, 5H, H2-Glc); 3.10 (bs, 10H); 2.56–2.54 (m, 5H, H3a-Neu5Ac); 2.45–2.42 (m, 10H, triazole-CH₂CH₂CH₂); 1.92 (s, 15H, NC(O)CH₃-Neu5Ac); 1.89 (s, 15H, NC(O)CH₃-GalNAc); 1.85–1.80 (m, 5H, H3b-Neu5Ac); 1.44–1.35 (m, 20H, CH₂ aliphatic chain); 1.14–1.08 (m, 10H, CH₂CH₂OC1-Glc), 1.03 (m, 40H, CH₂ aliphatic chain). ¹³C NMR (151 MHz, D₂O): δ (ppm) 175.4, 175.1, 174.5 (C(O)); 169.1 (ArC(O)NH); 159.7 (*Ar-ips*o); 134.8 (*Ar-ortho*); 129.2 (*Ar-para*); 128.9 (*Ar-meta*); 123.7 (C5 triazole); 105.1 (C1-Gal'); 103.0 (C1-Gal); 102.8 (C1-GalNAc); 102.5 (C1-Glc); 102.0 (C2-Neu5Ac); 80.7 (C3-GalNAc); 79.0 (C4-Glc); 77.6 (C4-Gal); 75.2 (C5-Gal'); 75.1 (C5-Glc); 74.9 (C3-Gal); 74.7; 74.4 (C5-Gal); 73.4 (C6-Neu5Ac); 73.1 (C2-Glc); 72.8 (C3-Gal'); 72.6 (C7-Neu5Ac); 71.0 (C2-Gal'); 70.9 (β-COCH₂); 70.4 (C2-Gal); 70.1–69.8; 69.4; 69.2; 69.0; 68.9; 68.4; 68.3 (C4-GalNAc); 63.1; 61.4; 61.3; 60.9; 60.5; 52.0; 51.5 (C2-GalNAc); 50.3; 40.0; 37.2; 31.1 (ArCH₂Ar); 29.3, 29.1, 29.0, 28.9, 28.8 (CH₂ aliphatic chain); 25.6 (CH₂CH₂OC1-Glc); 25.0 (triazole-CH₂CH₂CH₂); 23.0 (NHC(O)CH₃-GalNAc); 22.4 (NHC(O)CH₃-Neu5Ac). HR-ESI-MS(–): *m/z* 1600.5013 [100% (M – 5H)^{5–}] calcd: 1600.7112.

5,11,17,23,29-Pentaformyl-31,32,33,34,35-pentahydroxycalix[5]arene (3)

Calix[5]arene 2²⁵ (0.41 g, 0.78 mmol) was added to a solution of HMTA (2.5 g, 17.8 mmol) in 50 mL TFA and the mixture was refluxed for 5 days under N₂. The solvent was then removed under reduced pressure and the residue dissolved in 12 mL of a 1 : 1 CH₂Cl₂–HCl 1 M solution. The mixture was stirred at room temperature for 24 h. The aqueous phase was extracted



with CH_2Cl_2 (5×5 mL). The combined organic phases were washed with water (2×10 mL), dried over anhydrous Na_2SO_4 , filtered and the solvent removed under vacuum. The residue was purified by trituration in CHCl_3 –hexane 1:1 to give the product **3** as a brownish solid. Yield: 57%. ^1H NMR (300 MHz, CDCl_3 – CD_3OD 9:1): δ (ppm) 9.75 (s, 5H, CHO); 7.70 (s, 5H, ArOH); 7.22 (s, 10H, ArH); 3.45 (s, 10H, ArCH_2Ar). ^{13}C NMR (75 MHz, CDCl_3 – CD_3OD 9:1): δ (ppm) 192.1 (CHO); 150.6 (Ar-*ipso*); 131.9 (Ar-*para*); 128.1 (Ar-*meta*); 127.2 (Ar-*ortho*); 31.4 (Ar CH_2Ar). HR-ESI-MS(+): m/z 671.1921 [100% (M + H) $^+$] calcd: 671.1917. M.p. > 300 °C.

5,11,17,23,29-Pentaformyl-31,32,33,34,35-pentamethoxy-calix[5]arene (4)

In a two-neck round-bottomed flask, pentaformylcalix[5]arene **3** (0.7 g, 1.1 mmol) was dissolved in 150 mL of dry CH_3CN , then K_2CO_3 (4.5 g, 32 mmol) and CH_3I (2 mL, 32 mmol) were added and the mixture was refluxed for 20 h under a nitrogen atmosphere. The solvent was removed under reduced pressure and the residue dissolved in 150 mL of a 1:1 solution CH_2Cl_2 –HCl 1 M. The mixture was stirred for 2 h at room temperature. The organic layer was separated, and the aqueous phase extracted with CH_2Cl_2 (2×50 mL). The combined organic phases were washed with water (2×100 mL), dried over anhydrous Na_2SO_4 , filtered and the solvent removed under reduced pressure. The product **4** was obtained as a brown solid. Yield: 68%. ^1H NMR (300 MHz, CDCl_3): δ (ppm) 9.72 (s, 5H, CHO); 7.50 (s, 10H, ArH); 3.92 (s, 10H, ArCH_2Ar); 3.25 (s, 15H, OCH_3). ^{13}C NMR (75 MHz, CDCl_3 – CD_3OD 9:1): δ (ppm) 191.4 (CHO); 161.8 (Ar-*ipso*); 134.8 (Ar-*para*); 132.0 (Ar-*ortho*); 130.8 (Ar-*meta*); 60.6 (OCH_3); 30.7 (Ar CH_2Ar). HR-ESI-MS(+): m/z 741.2700 [100% (M + H) $^+$] calcd: 741.2700. M.p.: 217–219 °C.

5,11,17,23,29-Pentacarboxy-31,32,33,34,35-pentamethoxycalix[5]arene (5)

Pentaformyl-pentamethoxycalix[5]arene **4** (0.25 g, 0.34 mmol) was dissolved in a two-neck round-bottomed flask in 100 mL of a mixture acetone– CHCl_3 1:1, and cooled to 0 °C with an ice-water bath. In another flask, a solution of NaClO_2 80% pure (0.47 g, 4.20 mmol) was dissolved in the minimum amount of water. Subsequently, sulfamic acid (0.49 mg, 5.04 mmol) was added. This solution was slowly poured into the reaction flask. The mixture was stirred at 0 °C for 15 min and gradually warmed to room temperature while it remained stirred for 24 h. The solvent was then removed under reduced pressure and the residue triturated with 1 M HCl. After filtration on a Büchner funnel, product **5** was obtained as a solid. Yield: 79%. ^1H NMR (300 MHz, CD_3OD): δ (ppm) 7.75 (s, 10H, ArH); 3.93 (s, 10H, ArCH_2Ar); 3.30 (s, 15H, OCH_3). ^{13}C NMR (25 MHz, CD_3OD): δ (ppm) 169.6 (CO); 162.1 (Ar-*ipso*); 135.8 (Ar-*ortho*); 132.0 (Ar-*meta*); 126.8 (Ar-*para*); 61.3 ($-\text{OCH}_3$); 31.9 (Ar CH_2Ar). HR-ESI-MS(+): m/z 843.2269 [100% (M + Na) $^+$] calcd: 843.2265.

5,11,17,23,29-Pentakis[(17-azide-3,6,9,12,15-pentaoxahepta-decane-1-amino)carbonyl]-31,32,33,34,35-pentamethoxy-calix[5]arene (8)

In a round-bottomed flask, 0.12 g of calix[5]arene **5** (0.14 mmol) and 0.51 mL of oxalyl chloride (5.82 mmol) were solubilized in 15 mL of dry CH_2Cl_2 under a nitrogen atmosphere. The solution was stirred for 18 h at room temperature and then the solvent evaporated to dryness. The residual compound **6** was dissolved again in 5 mL of dry CH_2Cl_2 and then added dropwise to a solution of amine compound **7** (0.29 g, 0.87 mmol) and NEt_3 (0.12 mL, 0.87 mmol) in 5 mL of dry CH_2Cl_2 . The mixture was stirred for 20 h at room temperature under a nitrogen atmosphere. The mixture was then washed with 1 M HCl, an aqueous solution of Na_2CO_3 and water till neutral pH was reached. The solvent was removed under vacuum and the crude purified by flash chromatography (eluent: CHCl_3 – CH_3OH 95:5) to give the product **8** as a yellow oil. Yield: 44%. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 7.50 (s, 10H, ArH); 7.07 (bs, 5H, CONH); 3.89 (s, 10H, ArCH_2Ar); 3.65–3.50 (m, 110H, OCH_2 , CH_2NHCO); 3.34 (t, 10H, $J = 4.8$ Hz, CH_2N_3); 3.28 (s, 15H, OCH_3). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 167.3 (CO); 159.2 (Ar-*ipso*); 134.2 (Ar-*para*); 129.8 (Ar-*ortho*); 128.2 (Ar-*meta*); 70.6, 70.5, 70.2, 70.0, (OCH_2); 60.8 (OCH_3); 50.6 (CH_2N_3); 39.7 (CH_2NHCO); 30.9 (Ar CH_2Ar). HR-ESI-MS(+): m/z 1131.5698 [100% (M + 2H) $^{2+}$]; calcd: 1131.5756.

GM2-calix[5]arene (11)

Calix[5]arene **8** (15.7 mg, 6.93 μmol) was dissolved in 0.5 mL of CH_3OH in a microwave tube. Then the GM2os derivative **10** (50.5 mg, 52.1 μmol), previously synthesized by chemo-enzymatic procedures,^{30,34} was added together with samples of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.52 mg, 2.1 μmol), sodium ascorbate (0.82 mg, 4.2 μmol), 4 mL of H_2O and a drop of Triton X-100. The mixture was heated at 80 °C by microwave irradiation (150 W) for 60 min. The reaction progress was monitored *via* TLC (eluent: AcOEt – CH_3OH – H_2O – AcOH 4:2:1:0.1) and ESI-MS analyses. The crude material was purified *via* size exclusion column chromatography (Sephadex G-15, eluent: H_2O 100%), followed by HPLC purification (see General information) giving pure product **11** as a white solid. Yield: 59%. ^1H NMR (400 MHz, $\text{D}_2\text{O}/\text{CD}_3\text{OD}$): δ (ppm) 7.65 (s, 5H, H5 triazole); 7.54 (s, 10H, ArH); 4.70 (m, 5H, H1-Gal (determined by HSQC)); 4.47–4.42 (m, 15H, H1-GalNAc); 4.36–4.35 (d, 5H, $J = 7.6$ Hz, H1-Glc); 4.08–4.06 (m, 10H); 3.89–3.66 (m, 95H, ArCH_2Ar); 3.58–3.43 (m, 150H); 3.32–3.30 (m, 5H, H2-Gal); 3.23–3.19 (m, 5H, H2-Glc); 3.14 (bs, 10H); 2.61–2.58 (m, 5H, H3a-Neu5Ac); 2.48 (bs, 10H); 1.97 (s, 15H, NHC(O)CH_3 -Neu5Ac); 1.95 (s, 15H, NHC(O)CH_3 -GalNAc); 1.89–1.84 (m, 5H, H3b-Neu5Ac); 1.51–1.36 (bs, 20H, CH_2 aliphatic chain); 1.23–0.95 (50H, m, CH_2 aliphatic chain). HR-ESI-MS(–): m/z 1440.4670 [100% (M – 5H) $^{5-}$] calcd: 1438.6588.

Peracetylated-galactosylcalix[5]arene (14)

Calix[5]arene **8** (32.0 mg, 14.1 μmol) and the β -galactoside derivative **12** (52.9 mg, 106 μmol) were dissolved in 2.5 mL of DMF in a microwave tube. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (2.0 mg, 8.5 μmol),



sodium ascorbate (3.3 mg, 16.9 μmol) and 0.5 mL H_2O were then added. The mixture was heated at 80 $^\circ\text{C}$ by microwave irradiation (150 W) for 60 min. When the reaction was completed (checked *via* TLC, eluent: CH_2Cl_2 - CH_3OH 20 : 1), it was quenched by addition of water (15 mL) and extracted with AcOEt (5 \times 15 mL). The combined organic layers were dried over anhydrous Na_2SO_4 , filtered and the solvent removed under vacuum. The crude material was purified on preparative TLC plates (eluent: CH_2Cl_2 - CH_3OH 9 : 1) giving product **14** as a yellow oil. Yield: 67%. ^1H NMR (300 MHz, CD_3OD): δ (ppm) 8.21 (bs, 5H, C(O)NH); 7.77 (s, 5H, H5 triazole); 7.61 (bs, 10H, ArH); 5.38 (d, 5H, $J = 2.7$ Hz, H4); 5.16–5.02 (m, 10H, H3, H2); 4.61 (d, 5H, $J = 7.3$ Hz, H1); 4.51 (t, 10H, $J = 5.0$ Hz, OCH_2CH_2 -triazole); 4.20–4.05 (m, 15H, H5, H6a, H6b); 3.94 (bs, 10H, ArCH_2Ar); 3.88–3.77 (m, 15H, OCH_2CH_2 -triazole, β -COCHa); 3.70–3.44 (m, 105H, OCH2, β -COCHb, C(O)NHCH₂); 3.29 (s, 15H, ArOCH_3); 2.67 (t, 10H, $J = 7.6$ Hz, triazole- $\text{CH}_2\text{CH}_2\text{CH}_2$); 2.13 (s, 15H, Ac); 2.02 (s, 15H, Ac); 2.01 (s, 15H, Ac); 1.94 (s, 15H, Ac); 1.71–1.59 (m, 10H, triazole- $\text{CH}_2\text{CH}_2\text{CH}_2$), 1.59–1.47 (m, 10H, β -COCH₂CH₂), 1.40–1.23 (m, 50H, CH₂ aliphatic chain). ^{13}C NMR (75 MHz, CD_3OD) δ ppm: 172.0, 171.5, 171.2 (Ac); 169.6 (C(O)NH); 160.9 (Ar-*ipso*); 149.0 (C4 triazole); 135.8 (Ar-*ortho*); 130.7 (Ar-*para*); 129.7 (Ar-*meta*); 123.9 (C5 triazole); 102.2 (C1); 72.4 (C3); 71.7 (C5); 71.5, 71.4, 71.3, 70.9 (OCH₂); 70.6 (OCH₂CH₂-triazole); 70.5 (C2); 68.8 (C4); 62.6 (C6); 61.5 (ArOCH₃); 51.3 (OCH₂CH₂-triazole); 41.0 (C(O)NHCH₂); 32.0 (ArCH₂Ar); 30.6, 30.4, 30.2 (CH₂ aliphatic chain); 27.0 (triazole- $\text{CH}_2\text{CH}_2\text{CH}_2$); 26.3 (CH₂ aliphatic chain); 20.8, 20.6, 20.5 (CH₃C(O)). HR-ESI-MS(+): m/z 1607.7872 [100% (M + 3Na)³⁺] calcd: 1607.7813.

Peracetylated-lactosylcalix[5]arene (15)

Calix[5]arene **8** (27.5 mg, 12.0 μmol) and the β -lactoside compound **13** (70.0 mg, 89.0 μmol) were dissolved in 2.5 mL DMF in a microwave tube. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (2.6 mg, 10.4 μmol), sodium ascorbate (4.4 mg, 22.2 μmol) and 0.5 mL H_2O were then added. The mixture was heated at 80 $^\circ\text{C}$ by microwave irradiation (150 W) for 60 min. When the reaction was completed (checked *via* TLC, eluent: CH_2Cl_2 - CH_3OH 94 : 6), it was quenched by addition of water (15 mL) and extracted with AcOEt (5 \times 15 mL). The combined organic layers were dried over anhydrous Na_2SO_4 , filtered and the solvent removed *in vacuo*. The crude was purified by flash chromatography (elution in gradient: CH_2Cl_2 - CH_3OH 96 : 4 \rightarrow 95 : 5) giving product **15** as a yellow oil. Yield: 57%. ^1H NMR (300 MHz, CDCl_3): δ (ppm) 7.46 (bs, 10H, ArH); 7.40 (s, 5H, H5 triazole); 6.83 (bs, 5H, C(O)NH); 5.33 (d, 5H, $J = 3.3$ Hz, H4'); 5.17 (t, 5H, $J = 9.3$ Hz, H3); 5.09 (dd, 5H, $J_{1'-2'} = 7.9$ Hz, $J_{2'-3'} = 10.4$ Hz, H2'); 4.93 (dd, 5H, $J_{2'-3'} = 10.4$ Hz, $J_{3'-4'} = 3.3$ Hz, H3'); 4.86 (dd, 5H, $J_{2-3} = 9.3$ Hz, $J_{1-2} = 8.1$ Hz, H2); 4.50–4.38 (m, 25H, H1', H1, H6a, OCH₂CH₂-triazole); 4.15–4.00 (m, 15H, H6b, H6a', H6b'); 3.90–3.71 (m, 35H, H5', H4, β -COCHa, ArCH₂Ar, OCH₂CH₂-triazole); 3.65–3.47 (m, 105H, OCH₂, H5, C(O)-NHCH₂); 3.42 (m, 5H, β -COCHb); 3.21 (s, 15H, ArOCH₃); 2.65 (t, 10H, $J = 7.7$ Hz, triazole- $\text{CH}_2\text{CH}_2\text{CH}_2$); 2.12 (s, 15H, Ac); 2.09 (s, 15H, Ac); 2.04–1.98 (m, 60H, Ac); 1.94 (s, 15H, Ac);

1.65–1.55 (m, 10H, triazole- $\text{CH}_2\text{CH}_2\text{CH}_2$); 1.55–1.44 (m, 10H, β -COCH₂CH₂); 1.39–1.15 (m, 50H, CH₂ aliphatic chain). ^{13}C NMR (100 MHz, CDCl_3): δ ppm 170.4, 170.3, 170.2, 170.1, 169.8, 169.6, 169.1 (Ac); 167.3 (C(O)NH); 159.2 (Ar-*ipso*); 148.2 (C4 triazole); 134.2 (Ar-*ortho*); 129.9 (Ar-*para*); 128.2 (Ar-*meta*); 121.7 (C5 triazole); 101.1 (C1'); 100.6 (C1); 76.3 (C4); 72.8 (C3); 72.5 (C5); 71.7 (C2); 71.0 (C3'); 70.6 (C5'); 70.5 (OCH₂); 70.2 (β -COCH₂); 69.8 (OCH₂); 69.6 (OCH₂CH₂-triazole); 69.1 (C2'); 66.6 (C4'); 62.1 (C6); 60.8 (C6'); 60.7 (ArOCH₃); 50.0 (OCH₂CH₂-triazole); 39.7 (C(O)NHCH₂); 31.0 (ArCH₂Ar); 29.5, 29.3, 25.8, 25.7 (CH₂ aliphatic chain); 20.9, 20.8, 20.6, 20.5 (CH₃C(O)). HR-ESI-MS(+): m/z 1571.9445 [100% (M + 4Na)⁴⁺] calcd: 1571.940.

Galactosylcalix[5]arene (16)

The peracetylated-galactosylcalix[5]arene **14** (45.0 mg, 9.46 μmol) was dissolved in 5 mL of CH_3OH , drops of a freshly prepared MeONa in methanol solution were added till pH 8–9. The mixture was stirred at room temperature for 4 h. The progress of the reaction was monitored *via* ESI-MS analysis. Amberlite resin IR 120/H⁺ was subsequently added to quench the reaction, and the mixture was gently stirred for 30 min. until neutral pH was reached. The resin was then filtered off and the solvent removed under vacuum to give pure product **16** as a yellow oil. Yield. 90%. ^1H NMR (300 MHz, CD_3OD): δ (ppm) 8.23 (bs, 5H, C(O)NH); 7.77 (s, 5H, H5 triazole); 7.61 (s, 10H, ArH); 4.51 (t, 10H, $J = 5.0$ Hz, OCH₂CH₂-triazole); 4.20 (d, 5H, $J = 7.1$ Hz, H1); 3.94 (bs, 10H, ArCH₂Ar); 3.92–3.80 (m, 20H, H4, β -COCHa, OCH₂CH₂-triazole); 7.77–7.69 (m, 10H, H6a, H6b); 3.66–3.40 (m, 120H, β -COCHb, H2, H3, H5, OCH₂, C(O)-NHCH₂); 3.29 (s, 15H, OCH₃); 2.67 (t, 10H, $J = 7.6$ Hz, triazole- $\text{CH}_2\text{CH}_2\text{CH}_2$); 1.72–1.53 (m, 20H, triazole- $\text{CH}_2\text{CH}_2\text{CH}_2$, β -COCH₂CH₂); 1.44–1.24 (m, 50H, CH₂ aliphatic chain). ^{13}C NMR (75 MHz, CD_3OD): δ ppm 169.6 (C(O)NH); 160.9 (Ar-*ipso*); 149.0 (C4 triazole); 135.8 (Ar-*ortho*); 130.7 (Ar-*para*); 129.7 (Ar-*meta*); 124.0 (C5 triazole); 105.0 (C1); 76.6, 75.1, 72.6 (C2, C3, C5); 71.5, 71.4, 71.3, 70.8, 70.6, 70.4, 70.3 (OCH₂, β -COCH₂, C4); 62.5 (C6); 61.5 (ArOCH₃); 51.3 (OCH₂CH₂-triazole); 41.0 (C(O)NHCH₂); 32.0 (ArCH₂Ar); 30.8, 30.6, 30.5, 30.4, 30.3, 27.1 (CH₂ aliphatic chain); 26.3 (triazole- $\text{CH}_2\text{CH}_2\text{CH}_2$). HR-ESI-MS (+): m/z 1305.0569 [100% (M + 3H)³⁺] calcd: 1305.0601

Lactosylcalix[5]arene (17)

The peracetylated-lactosylcalix[5]arene **15** (42.0 mg, 6.8 μmol) was dissolved in 5 mL of CH_3OH , and drops of a freshly prepared methanol solution of MeONa were added till pH 8–9. The mixture was stirred at room temperature for 18 h. The progress of the reaction was monitored *via* ESI-MS analysis. Amberlite resin IR 120/H⁺ was subsequently added for quenching, and the mixture was gently stirred for 30 min till neutral pH. The resin was then filtered off and the solvent removed under vacuum to give pure product **17** as a yellow oil. Yield: 72%. ^1H NMR (300 MHz, CD_3OD): δ (ppm) 7.79 (s, 5H, H5 triazole); 7.60 (s, 10H, ArH); 4.51 (t, 10H, $J = 5.0$ Hz, OCH₂CH₂-triazole); 4.35 (d, 5H, $J = 7.3$ Hz, H1'); 4.26 (d, 5H, $J = 7.8$ Hz, H1); 3.93 (bs, 10H, ArCH₂Ar); 3.90–3.65 (m, 40H, H4', H6ab', H6ab, Glc β -COCHa, OCH₂CH₂-triazole); 3.65–3.35 (m, 135H, H3, H4,



H5, H2', H3', H5', Glc β -COCHb, OCH₂, C(O)NHCH₂; 3.28 (s, 15H, OCH₃); 3.23 (t, 5H, $J = 8.4$ Hz, H2); 2.66 (t, 10H, $J = 7.6$ Hz, triazole-CH₂CH₂CH₂); 1.69–1.53 (m, 20H, CH₂ aliphatic chain); 1.42–1.23 (m, 50H, CH₂ aliphatic chain). ¹³C NMR (75 MHz, CD₃OD): δ ppm 169.6 (C(O)NH); 160.9 (Ar-*ipso*); 148.8 (C4 triazole); 135.7 (Ar-*ortho*); 130.7 (Ar-*para*); 129.7 (Ar-*meta*); 124.2 (C5 triazole); 105.1 (C1'); 104.2 (C1); 80.7 (C4); 77.0, 76.5, 76.4, 74.8, 74.7, 72.5 (C2, C3, C5, C2', C3', C5'); 71.5, 71.4, 71.3, 70.9, 70.6, 70.4 (OCH₂, β -COCH₂); 70.3 (C4'); 62.5, 61.9 (C6, C6'); 61.5 (ArOCH₃); 51.4 (OCH₂CH₂-triazole); 41.0 (C(O)NHCH₂); 32.0 (ArCH₂Ar); 30.8, 30.6, 30.5, 30.4, 30.2, 27.1 (CH₂ aliphatic chain); 26.2 (triazole-CH₂CH₂CH₂). HR-ESI-MS (+) m/z : 1575.1475 [100% (M + 3H)³⁺] calcd: 1575.1481.

17-Azide-3,6,9,12,15-pentaoxaheptadecane-1-aminocarbonyl-*p*-methoxybenzene (19)

Oxalyl chloride (1.5 mL, 16.0 mmol) was added to a solution of 4-methoxybenzoic acid (0.30 g, 2.0 mmol) in 15 mL of dry CH₂Cl₂ and the mixture was stirred at room temperature under N₂ for 18 h. The solvent was then removed under vacuum and the residue dissolved again in 5 mL of dry CH₂Cl₂. This solution was added dropwise to a round bottomed flask containing the azidoamine compound **7** (0.91 g, 3.0 mmol) and NEt₃ (0.5 mL, 3.0 mmol) in 10 mL of dry CH₂Cl₂. The mixture was let to react for 20 h at room temperature under an N₂ atmosphere. The reaction was monitored *via* TLC (eluent: AcOEt). A 1 M HCl solution (20 mL) was then added to quench the reaction, and the product extracted with CH₂Cl₂ (2 \times 20 mL). The combined organic phases were washed with NaHCO₃ saturated aqueous solution (15 mL), brine (15 mL), water (15 mL), dried over anhydrous Na₂SO₄, filtered and the solvent evaporated under reduced pressure. The crude was purified by flash chromatography (eluent: AcOEt–acetone 9 : 1). Product **19** was obtained pure as a yellow oil. Yield: 20%. ¹H NMR (300 MHz, CDCl₃): δ (ppm) 7.74 (d, 2H, $J = 8.9$ Hz, Ar-*meta*); 6.87 (d, 2H, $J = 8.9$ Hz, Ar-*ortho*); 6.78 (bs, 1H, C(O)NH); 3.80 (s, 3H, OCH₃); 3.65–3.54 (m, 22H, OCH₂, C(O)NHCH₂); 3.32 (t, 2H, $J = 5.0$ Hz, CH₂N₃). ¹³C NMR (75 MHz, CDCl₃): δ ppm 167.0 (Ac); 162.0 (Ar-*ipso*); 128.8 (Ar-*meta*); 126.9 (Ar-*para*); 113.6 (Ar-*ortho*); 70.6, 70.5, 70.2, 70.0, 69.9 (OCH₂); 55.4 (OCH₃); 50.6 (CH₂N₃); 39.7 (C(O)NHCH₂). ESI-MS(+) m/z : 463.0 [100% (M + Na)⁺]; 435.0 [60% (M – N₂ + Na)⁺].

GM1os-monomer (20)

Starting from compounds **19** and **9**, following the same procedure as for compound **1**, and using reversed phase column chromatography for the purification (gradient MeOH–H₂O–AcOH), monomer **20** was obtained as a white solid in 49% yield. ¹H NMR (400 MHz, D₂O): δ (ppm) 7.81 (3H, m, triazole, Ar), 6.98 (2H, d, $J = 8.4$ Hz, Ar), 4.71 (1H, s, H1-GalNAc), 4.39 (1H, d, $J = 8.4$ Hz, H1-Gal'), 4.42 (1H, m, H1-Gal), 4.38 (1H, m), 4.31, (1H, d, $J = 8.2$ Hz, H1-Glc), 4.05–4.01 (3H, m), 3.93 (1H, m, H2-GalNAc), 3.75–3.40 (21H, m), 3.60–3.47 (16H, m), 3.36–3.28 (14H, m), 3.25 (1H, m, H2-Gal), 3.18 (1H, m, H2-Glc), 3.10 (2H, s), 2.54 (1H, m, H3a-Neu5Ac), 2.35 (2H, m, CH₂-triazole), 1.92 (3H, s, NHC(O)CH₃-Neu5Ac), 1.85 (3H, s, NHC-

(O)CH₃-GalNAc), 1.75 (1H, m, H3b-Neu5Ac), 1.40 (4H, m), 1.11 (2H, m, Glc β -COCH₂CH₂), 1.03 (8H, m, –CH₂CH₂CH₂–); ¹³C NMR (75 MHz, D₂O): δ (ppm) 174.8, 174.5, 173.9, 168.5, 159.2, 128.4 (2 \times CH Ar), 123.1 (C5 triazole), 113.2 (2 \times CH Ar) 104.6 (C1-Gal'), 102.7 (C1-Gal), 102.4 (C1-GalNAc), 102.0 (C1-Glc), 101.5 (C2-Neu5Ac), 80.2 (C3-GalNAc), 78.5 (C4-Glc), 77.0 (C4-Gal), 74.7, 74.6, 74.4, 74.2, 74.1, 74.0, 73.9 (C2 Glc), 72.6 (C3 Gal'), 72.3, 72.1 (C2-Gal'), 70.6 (Glc β -COCH₂), 70.4 (C2-Gal), 70.4–68.5 (–OCH₂–), 68.4 (C4-GalNAc), 67.8, 62.7, 60.9, 60.8, 60.4, 60.0, 51.4, 51.0 (C2 GalNAc), 49.8, 39.5, 36.7, 28.8–28.3 (CH₂CH₂CH₂), 25.0 (Glc β -COCH₂CH₂), 24.5 (CH₂-triazole), 22.5 (NHC(O)CH₃-GalNAc), 21.9 (NH(O)CH₃-Neu5Ac); HR-ESI-MS(–) m/z : 1587.7009 [100% (M – H)[–]] calcd: 1587.7034.

CTB5 inhibition assays

Each well of a 96-well microtiter plate was coated with a 100 μ L native GM1 solution (1.3 μ M in ethanol) after which the solvent was evaporated. Unattached GM1 was removed by washing with PBS (3 \times 450 μ L), the remaining free binding sites were blocked by incubation with 100 μ L of a 1% (w/v) BSA solution in PBS for 30 min at 37 °C. Detection limits were determined by placing a CT-horseradish peroxidase conjugate (CT-HRP), without inhibitor, on the plate, which gives the highest response, and the lowest response was determined by the optical density of the blank, *i.e.* the native GM1-coated well with all components except the inhibitor and the toxin. These two values represent the minimum and the maximum values of optical density, 0% and 100% of binding of the CT to the GM1-coating of the wells. Subsequently, the wells were washed with PBS (3 \times 450 μ L). In separate vials, a logarithmic serial dilution was performed that started from 2.0 mM of 150 μ L saccharide-calixarenes in 0.1% BSA and 0.05% Tween-20 in PBS. Next, each vial was mixed and incubated with 150 μ L of a 50 ng mL^{–1} CTB-HRP solution in the same buffer. This gave an initial inhibitor concentration of 1.0 mM. In the case of potent inhibitors, based on the logarithmic experiments, a more accurate, serial dilution of a factor two was performed around the expected IC₅₀-values. The inhibitor–toxin mixtures were incubated at room temperature for 2 h and then transferred to the coated wells. After 30 min of incubation at room temperature, unbound CTB-HRP-calixarene complexes were removed from the wells by washing with 0.1% BSA, 0.05% Tween-20 in PBS (3 \times 500 μ L). 100 μ L of a freshly prepared OPD solution (25 mg OPD-2HCl, 7.5 mL 0.1 M citric acid, 7.5 mL 0.1 M sodium citrate and 6 μ L of a 30% H₂O₂ solution, pH was adjusted to 6.0 with NaOH) was added to each well and allowed to react with HRP in the absence of light, at room temperature, for 15 minutes. The oxidation reaction was quenched by addition of 50 μ L 1 M H₂SO₄. Within 5 min, the absorbance was measured at 490 nm.

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